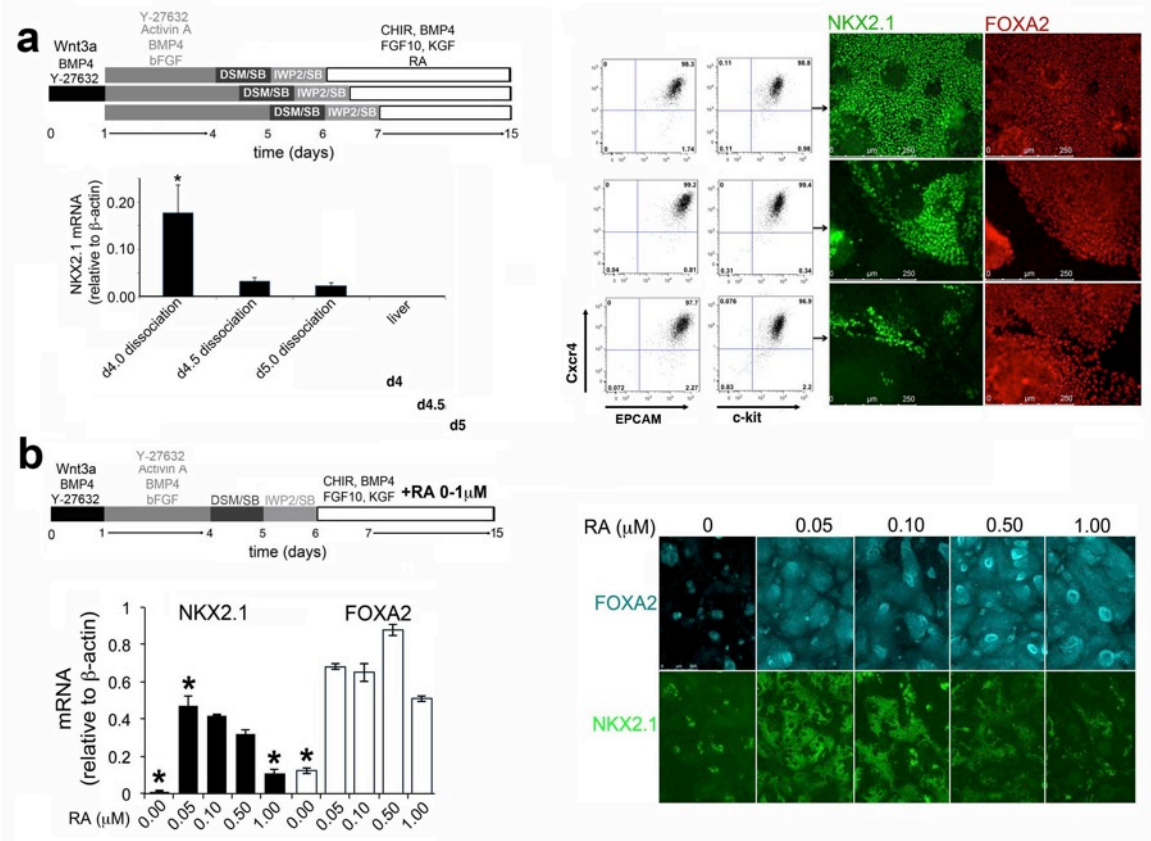
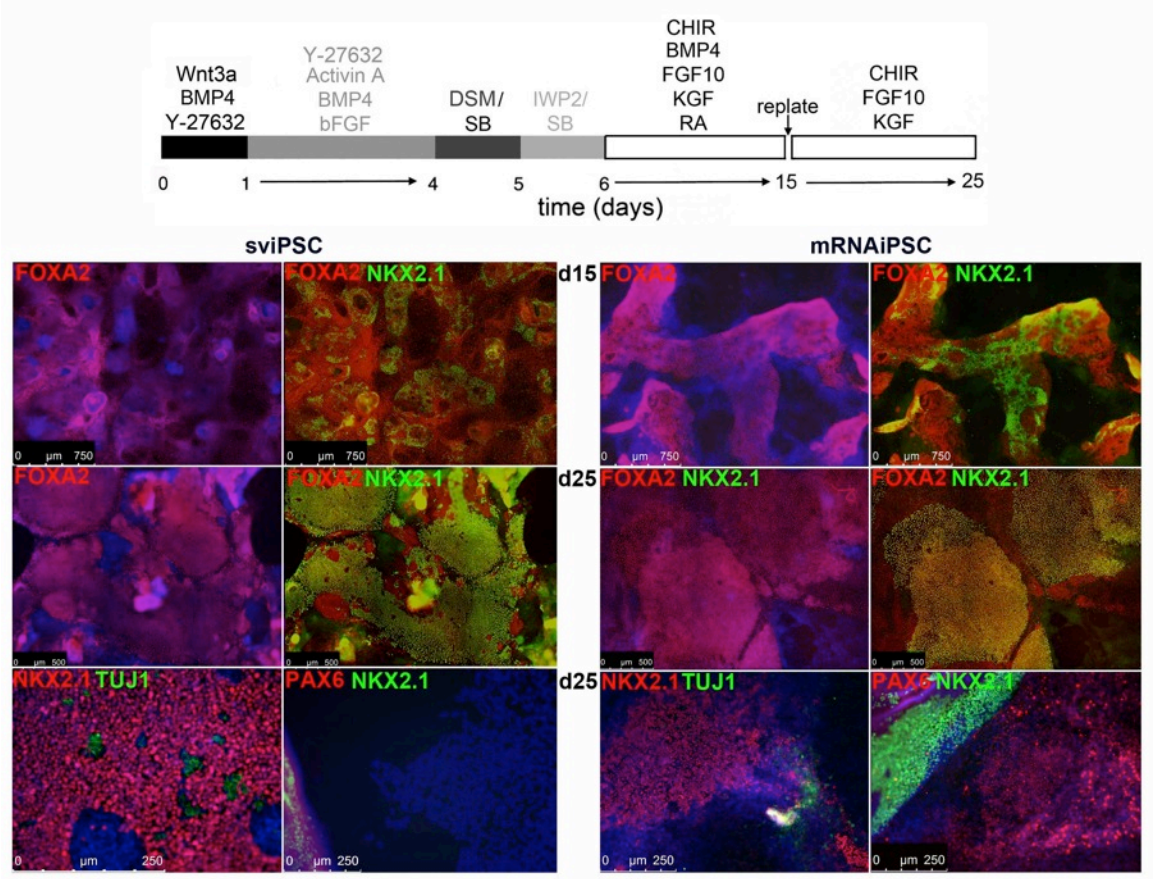
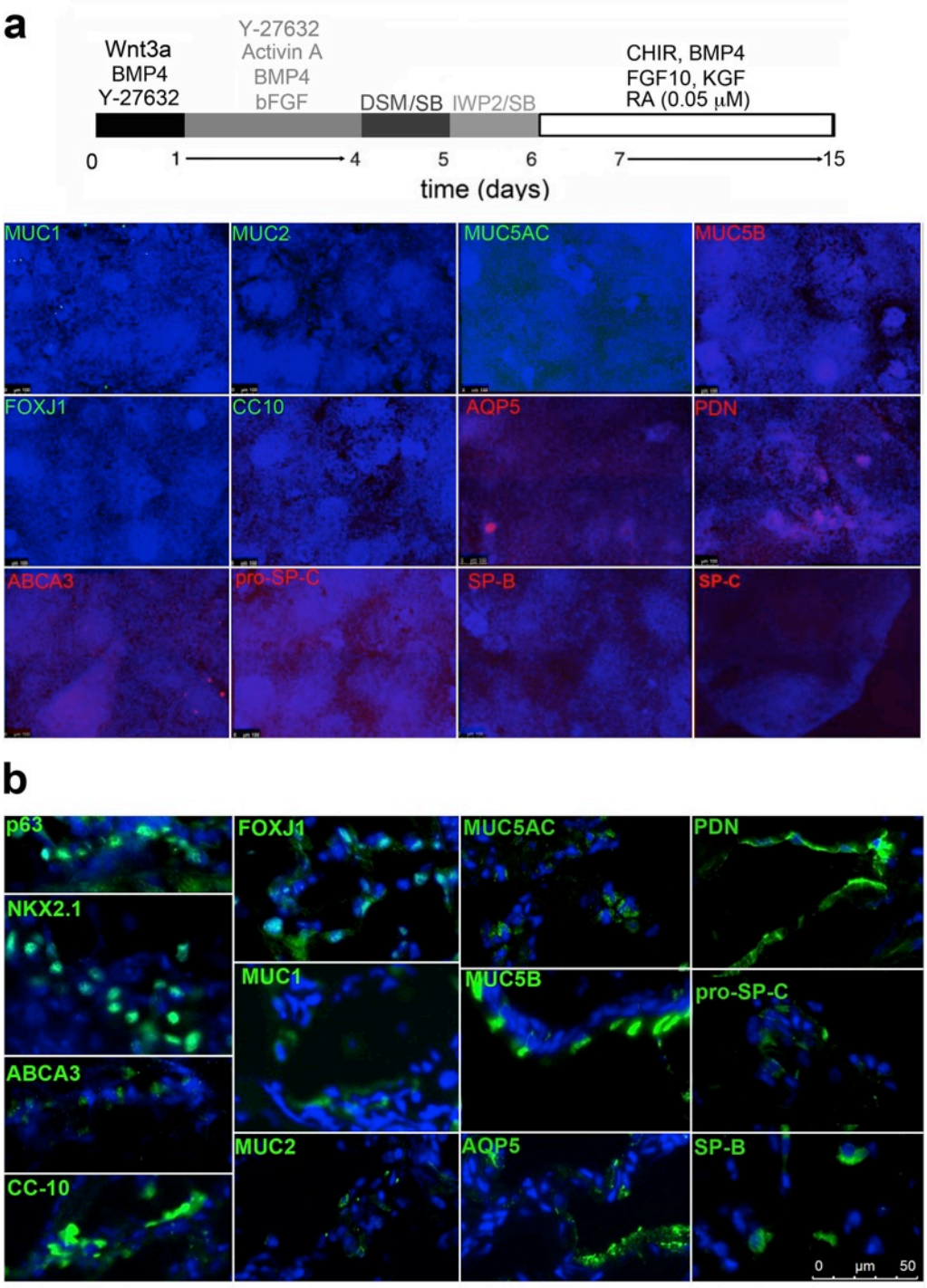
Supplementary Figures



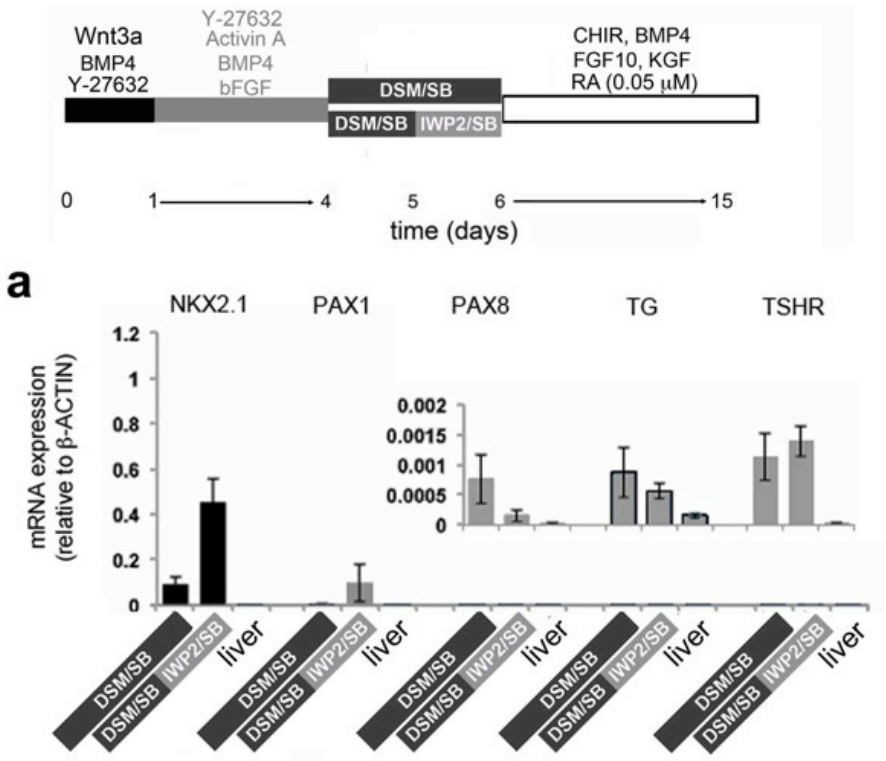
Supplementary Figure 1. Effect of timing of DE dissociation and RA concentration on lung field specification in hPSCs. (a) Effect of duration of endoderm induction on expression of NKX2.1 mRNA (left) and of NKX2.1 and FOXA2 protein (right) at d15 of the differentiation protocol shown on the upper left of the panel. The flow cytometric plots show the expression of the endoderm markers EPCAM, CXCR4 and c-KIT at the indicated time of dissociation of embryoid bodies. **P*<0.01, compared with d4.5 or d5 dissociations, triplicate experiments representative of 3 independent experiments. 'Liver' represents DE cultured in 'hepatic conditions'[1]. **(b)** Expression of NKX2.1 and FOXA2 mRNA (lower left) and protein (lower right) after culture of RUES2 cells according to the protocol on top of the panel using various concentrations of RA during the 'ventralization' stage. Triplicate experiments representative of 3 independent experiments, *, $P < 0.05$, compared with 0.5μM RA group.



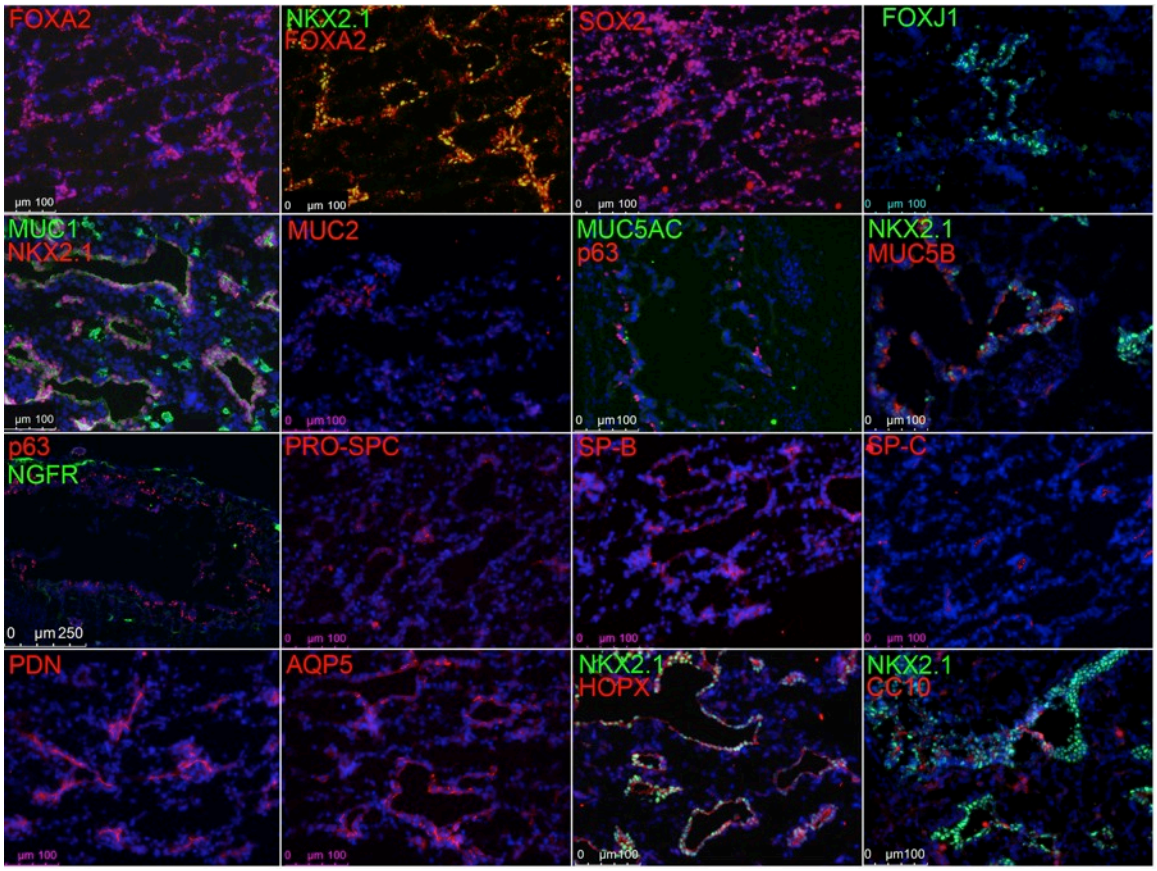
Supplementary Figure 2. Lung and airway progenitor differentiation from iPS cells. Fluorescence micrographs of expression of FOXA2 and NKX2.1 (d15, d25), and of TUJ1 and PAX6 (d25) in sviPSCs and mRNA iPSCs cultured according to the protocol shown on top of the figure. Representative of thee independent experiments.



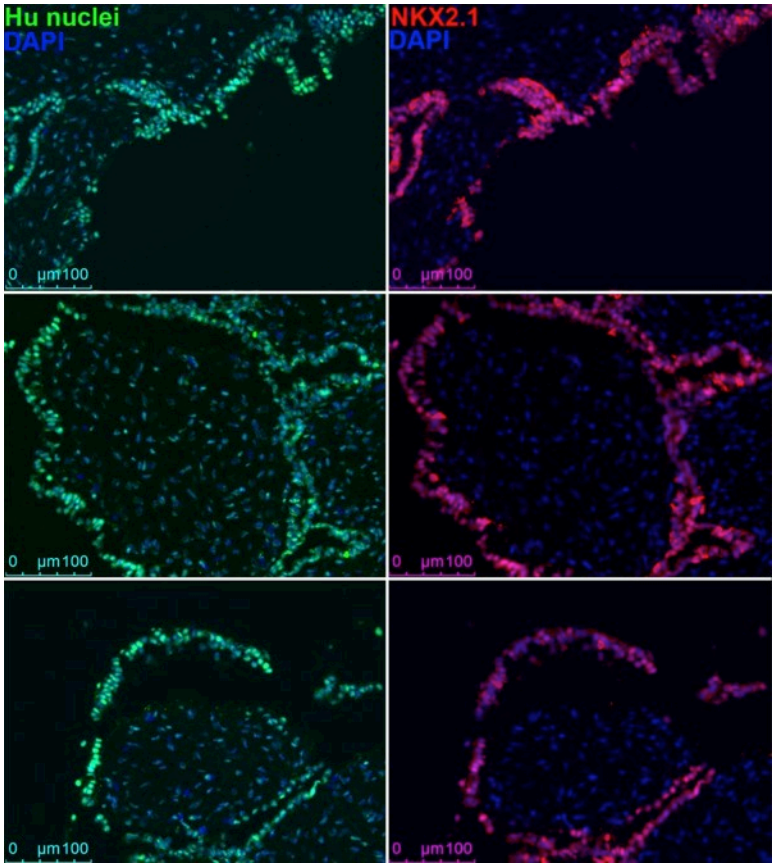
Supplementary Figure 3. Absence of expression of markers of lung and airway cells at d15 using AHL as positive staining controls **(a)** Representative example of the (absent) expression of markers of mature lung and airway epithelial cells after culturing RUES2 cells according to the protocol shown on top of the figure. Results representative of 4 independent experiments. **(b)** Fluorescence microscopy of frozen adult human lung sections stained with the indicated antibodies and with DAPI.



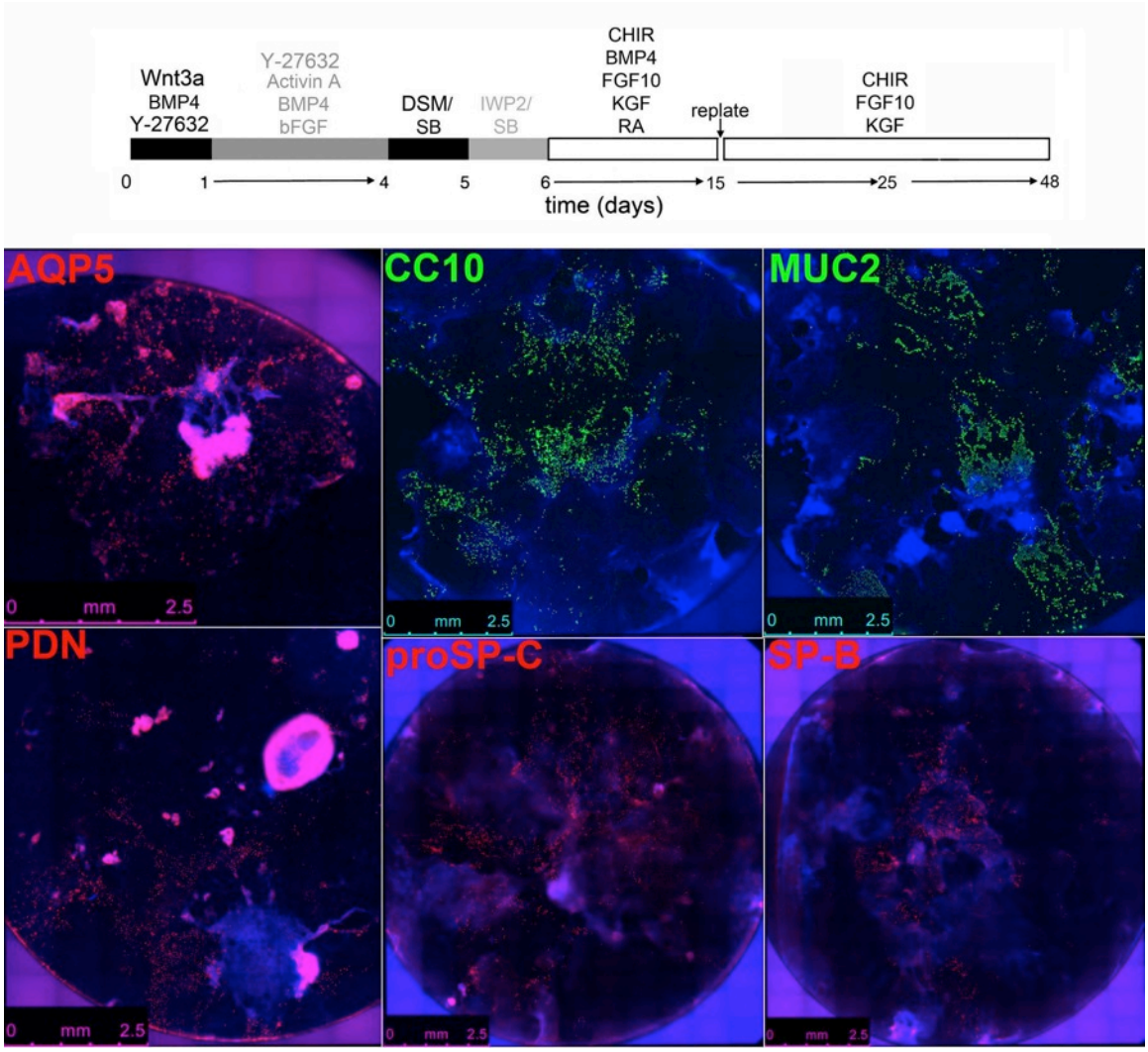
Supplementary Figure 4. Absence of thyroid gene expression in hPSCs-derived lung progenitor cultures. Expression of NKX2.1, PAX1, PAX8, TG (thyroglobulin) and TSHR mRNA at d15 in the two culture conditions shown on top of the figure. Triplicate experiment, representative of 3 independent experiments. 'Liver' represents DE cultured in the presence of BMP4 and bFGF, which specifies hepatic fate. The inset shows the expression of PAX8, TG and TSHR mRNA on a recalibrated Y-axis.



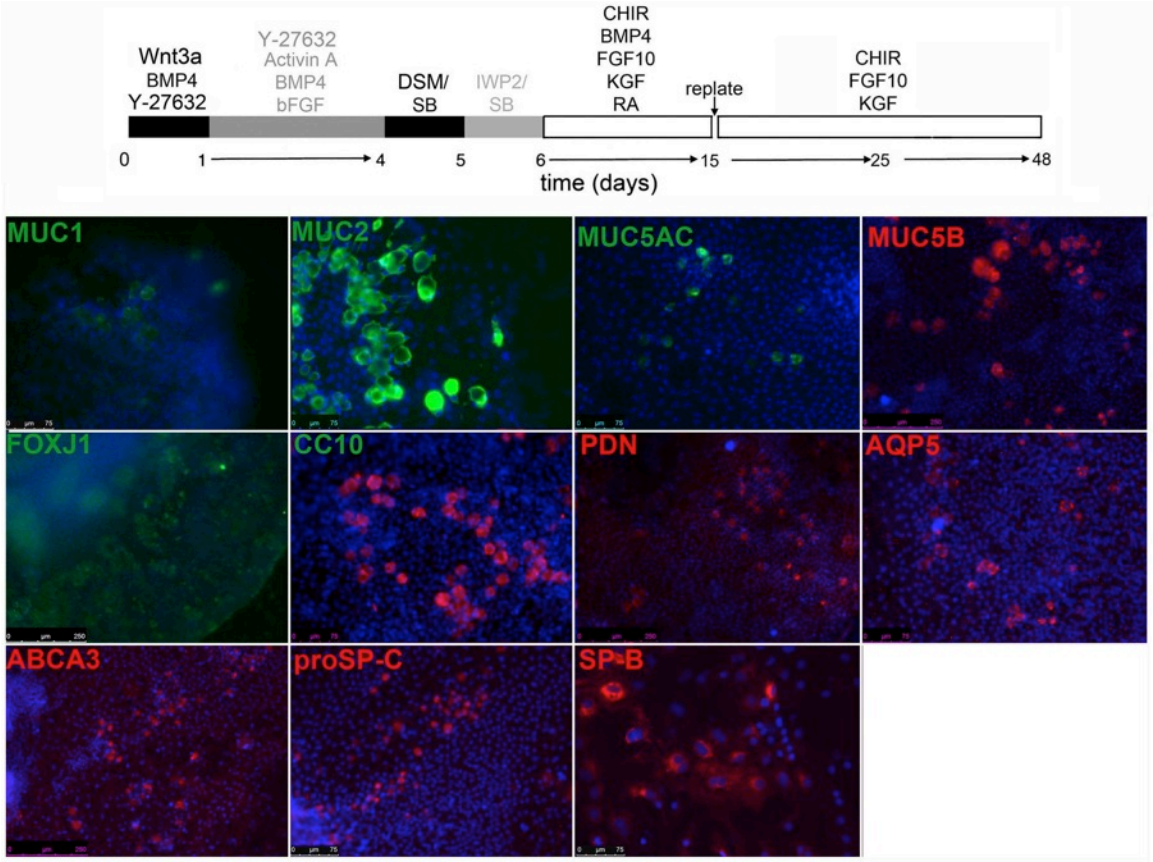
Supplementary Figure 5: Fluorescence microscopy of human fetal lung. Representative fluorescence micrographs of the expression of markers of lung and airway epithelium in midgestation human fetal lungs.



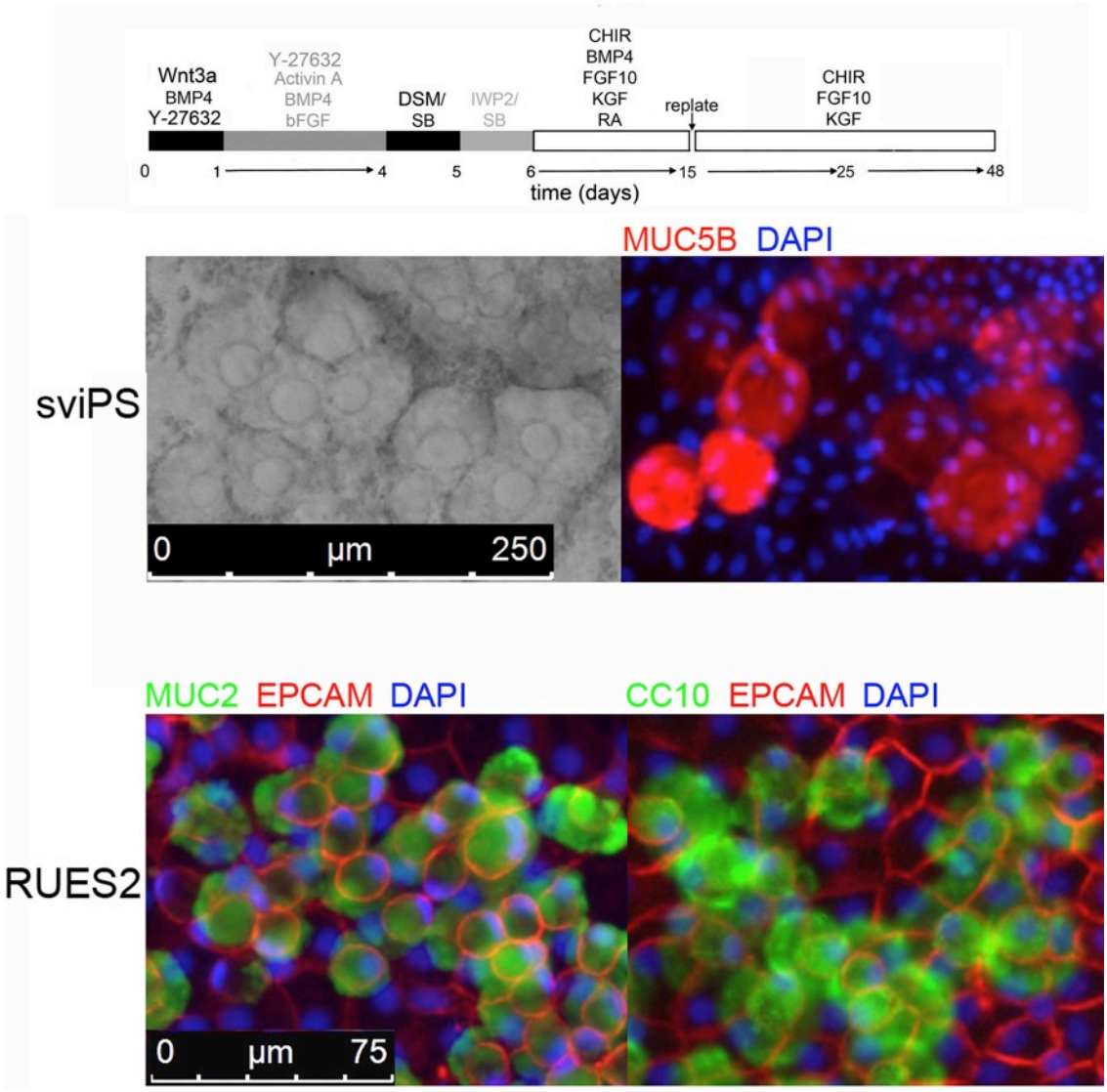
Supplementary Figure 6: Staining for human nuclei. Fluorescence micrographs of growths removed 6 months after transplantation of RUES2 cells differentiation according to the protocol shown in Fig. 1b, under the kidney capsule of NSG mice stained with antibodies against human nuclei and Nkx2.1.



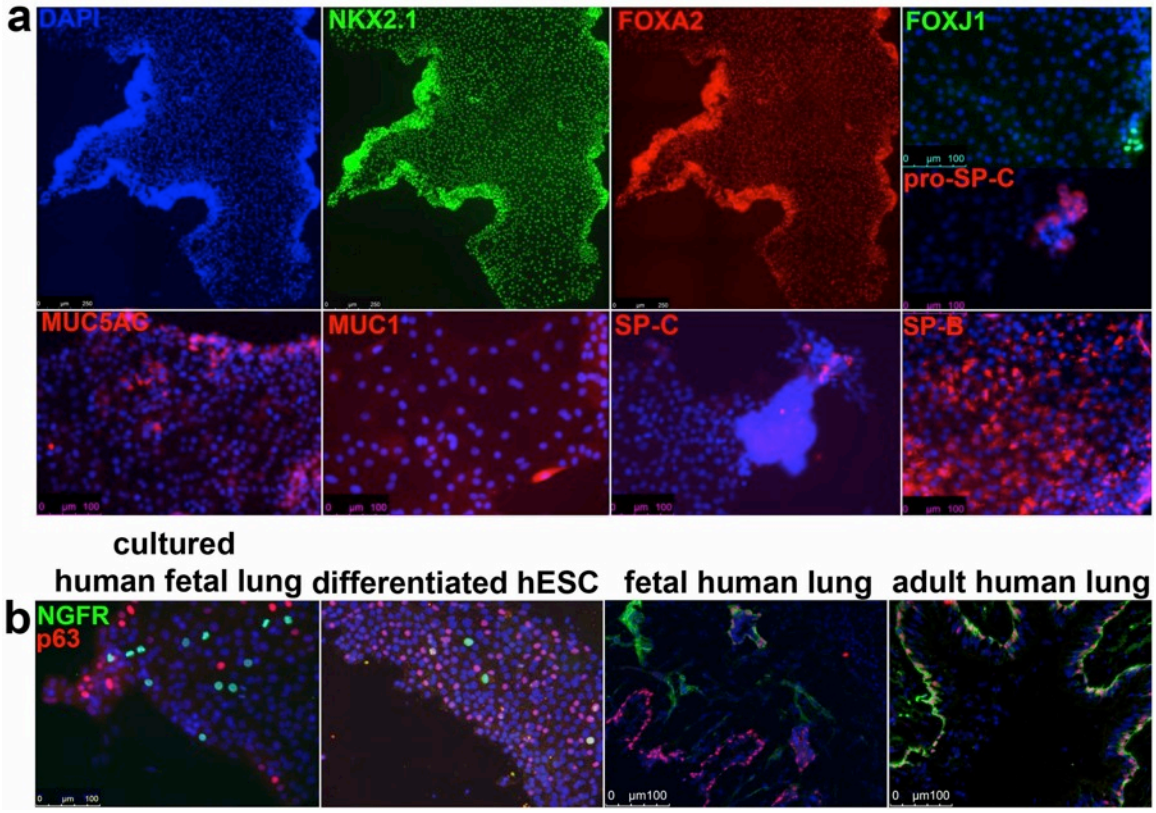
Supplementary Figure 7. Expression of markers of mature lung and airway epithelium in differentiated RUES2 cells. Representative 10x whole culture tile scans of RUES2 cells cultured according to the protocol shown on top of the figure. The densely pink areas in the PDN and AQP5 panels are staining artifacts.



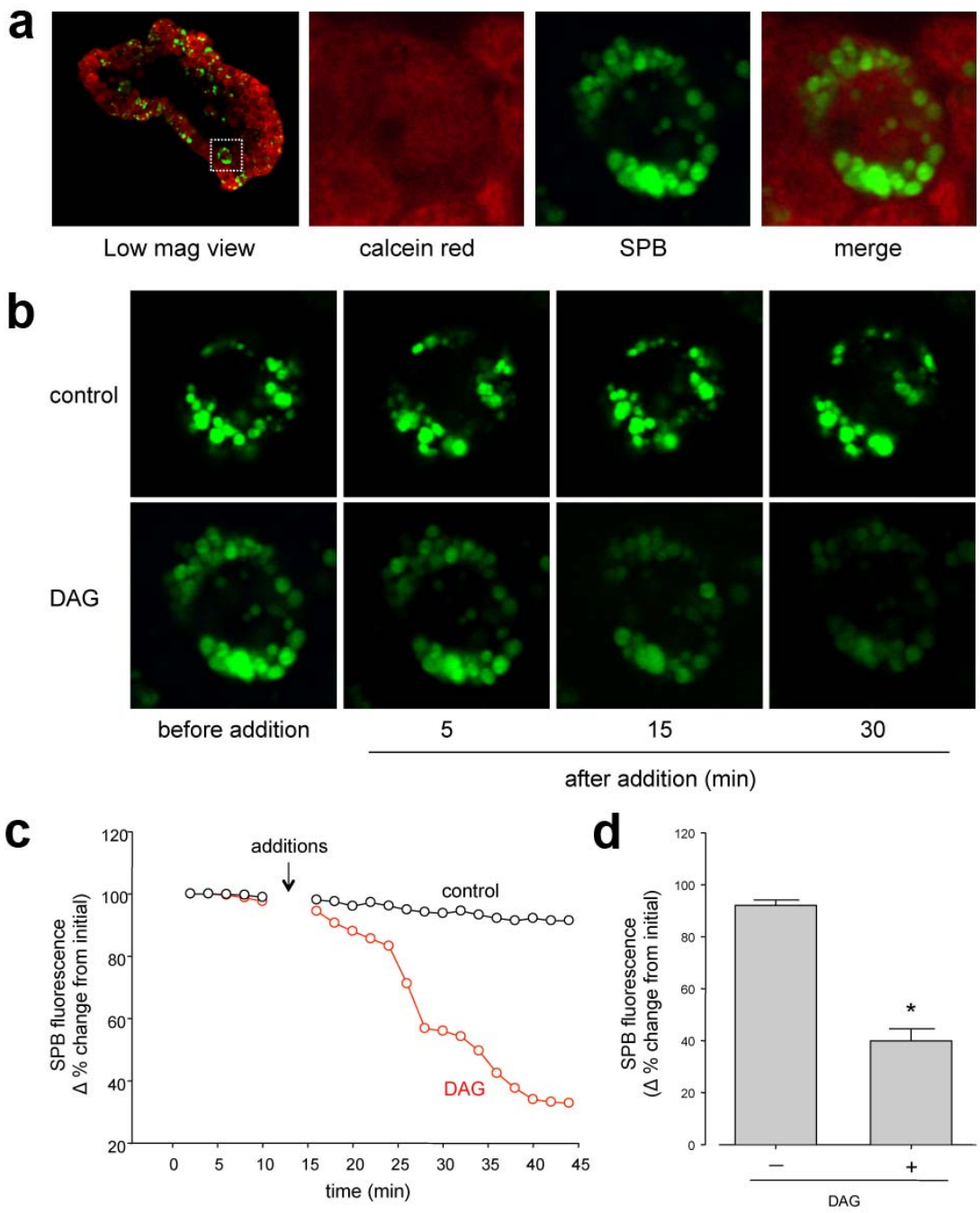
Supplementary Figure 8. Expression of markers of mature lung and airway epithelium in differentiated sviPSCs. Representative examples of the expression of markers of mature lung and airway epithelial cells after culturing sviPS cells according to the protocol shown on top of the figure. Immunofluorescence images represent reproducible results from 3 independent experiments.



Supplementary Figure 9. Morphological evidence for secretory activity. Large magnification images of staining of cultures of sviPS cells (top) and RUES2 cells (bottom) cultured according to the protocol on top of the figure for MUC5B, MUC2 and CC-10. RUES2 cells were also stained for EPCAM to determine cell boundaries. Bright field is shown for sviPS cells.



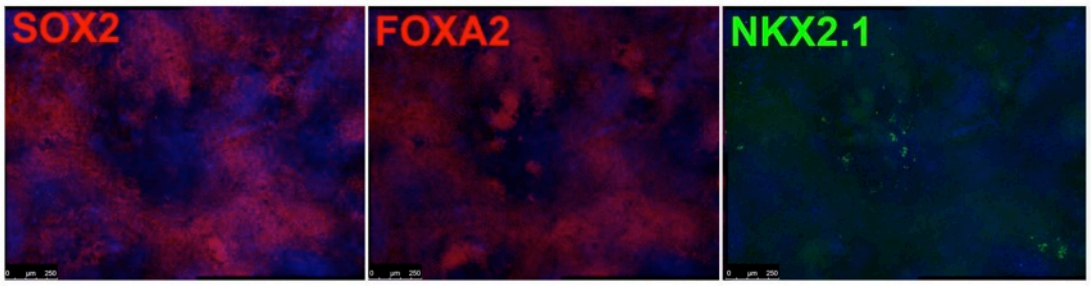
Supplementary Figure 10: Fluorescence microscopy of cultured fetal human lung. **(a)** Midgestation FHL was dissociated and cultured for 3 weeks in CHIR, FGF10 and KGF in the presence of DCI. **(b)** Expression of NGFR and p63 in cultured FHL cells, differentiated hESCs (d35), FHL and AHL.



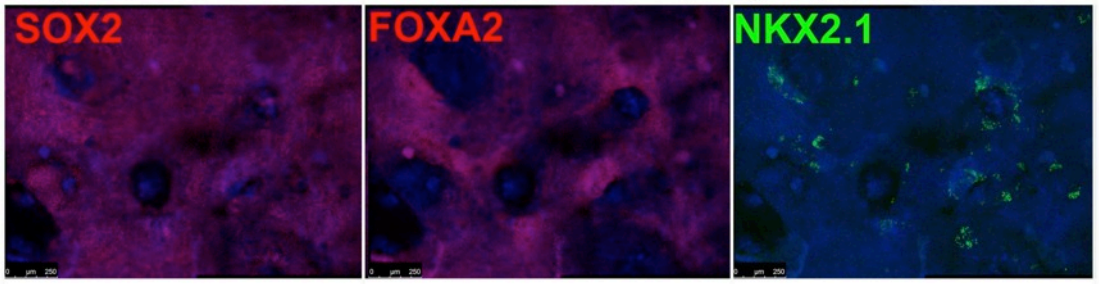
Supplementary Figure 11: Induction of BODIPY-SPB secretion. (a) Confocal images (optical section 1 μm) of uptake of fluorescently-labeled human SPB (BODIPY-SPB) by differentiated hPSCs. A select region (rectangle) in the low power view (left image) is magnified in the right images. The red and green channel renditions are shown respectively, the cytosol stained with calcein red and perinuclear lamellar bodies stained with BODIPY-SPB. **(b)** Confocal images of a single differentiated hPSC with lamellar

bodies stained with BODIPY-SPB. Images were taken sequentially after addition of the cell-permeable diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-sn-glycerol, or DMSO carrier control. **(c,d)** DAG-induced (arrow) surfactant secretion in one single cell (**c**) and average over 12 cells in each group (**d**). Surfactant secretion is denoted by a loss of fluorescence. n = 12 cells (from 3 independent experiments) in each group. *P < 0.05 compared to without DAG.

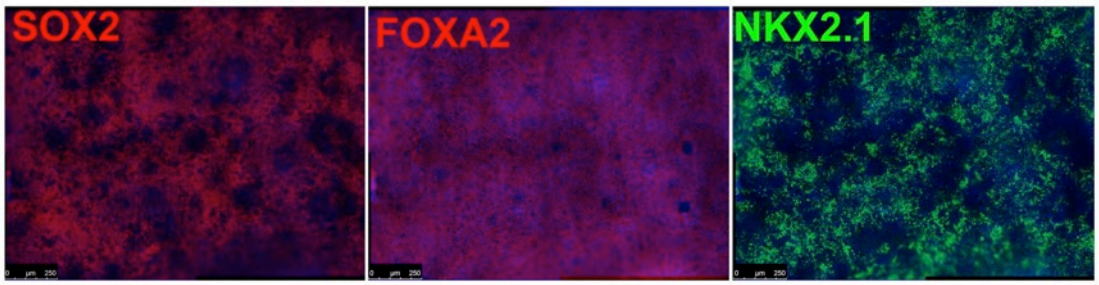
anteriorization: FGF2+SHH (Wong et al.)
lung field induction: FGF10+FGF7+BMP4 (5 ng/ml) (Wong et al)



anteriorization: DSM/SB>SB/I (Huang et al.)
lung field induction: FGF10+FGF7+BMP4 (5 ng/ml) (Wong et al)



anteriorization: FGF2+SHH (Wong et al.)
lung field induction: FGF10+FGF7+CHIR+BMP4 (50ng/ml)+RA (50nM)
(Huang et al)



Supplementary Figure 12. Comparison with the protocol of Wong et al. Lung progenitor generation from hPSCs using the protocol published by Wong et al (ref. 8). Lower panels show lung field generation using our anteriorization protocol and the lung field induction protocol of Wong et al., as well as the anteriorization protocol of Wong et al. and our lung field induction protocol.

Supplementary Experimental Procedures

Immunofluorescence

On d25 differentiation culture, "Nkx2.1(mouse)/p63/FOXA2" and "Nkx2.1(mouse)/Sox2/FOXA2" triple stains were processed as following: after blocking non-specific binding, the cultures were incubated in a cocktail of Nkx2.1 and Foxa2 primary antibodies for 12 hr at 4°C, followed by 3×10 min wash at room temperature, cultures were incubated in donkey anti-mouse Alexa Fluor 488 and donkey anti-goat Alexa Fluor 647 for 2 hr and washed 3 times. The cultures were then blocked in 5% fetal donkey serum for 2 hr at room temperature, followed by p63 primary antibody incubation for 12hr, after 3×10 min wash, the cells were stained with donkey anti-rabbit Cy3 for 2 hr at room temperature, washed and stained with DAPI and preserved in dark at 4°C for imaging.

"Tuj1/TTF-1(Rb)/FOXA2" triple stain was processed by incubating the cultures in Nkx2.1 and Foxa2 primary antibody cocktails for 12 hr at 4°C, wash 3×10 min at room temperature, donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 647 were added and incubated for 2 hr at room temperature, after 3 times washes, the cultures were then blocked in 5% fetal donkey serum overnight at 4°C, followed by 1hr Tuj1 primary antibody incubation at room temperature, the cultures were washed for additional 3 times, donkey anti-mouse Alexa Fluor 488 secondary was added for h hr at room temperature. The cultures were washed, incubated in DAPI and preserved in PBS for imaging.

Isolation and Culture of human fetal lung cells

Human fetal lungs (Gestational age of 22-23 weeks) were purchased from Advanced Bioscience Resources (INC, Alameda, CA). The tissues were sliced into small pieces, homogenized and passed through 100 μM cell strainers. The resulting cell suspensions were treated with RBC lysis buffer (affymetrix/eBioscience, San Diego, CA) for 5min and washed twice with DPBS, suspended in SFD media containing CHIR99021, 3μM; human FGF10, 10 ng/ml; human FGF7, 10 ng/ml onto 10mm tissue culture dishes. The non-epithelial cells attached to the bottom of the dish within 24hr, small cell mass formed by epithelial cells appear as suspension in the media. 48hr later, the suspension epithelial cell mass were collected and plated onto new fibronectin-coated tissue culture plates or 35mm culture dishes in SFD media containing CHIR99021, human FGF10, and human FGF7. Allow the cell mass to attach within a few days. On day 6-post isolation, maturation components were added in SFD media in the presence of CHIR99021 human FGF10, and human FGF7. The cultures were maintained for additional 21 days before being processed for immunofluorescence or electron microscopy.

Live cell imaging of fluorescent-SPB protein secretion in d48 cultures

For assaying the secretion of fluorescent-SP-B protein by day48 cultures, the cells were incubated with BODIPY-SPB as described in online methods, the cultures were counter stained with CellTrace calcein red-orange AM (Life Technologies) for 10min, the baseline BODIPY-SPB fluorescence were imaged for 20min with Zeiss LSM 510 confocal microscope, the cells were then incubated in 10μM of a DAG analogue, 1-Oleoyl-2-acetyl-sn-glycerol (Sigma, cat# O6754) for 10min. Cultures were rinsed with DPBS and imaged for another 20min. The minus DAG controls were processed identically and imaged for the same length of time. The fluorescent intensity of chosen cells was analyzed with Metamorph (Molecular Devices, LLC. Sunnyvale, CA).

Supplementary tables

Supplementary table I: Immunofluorescence Staining Antibodies

Antibody name	Host species	Clone number	Manufacturer	Catalog number	Dilution Factor
Foxa2/HNF-3β	Goat	M-20	Santa Cruz	sc-6554	1:50
Nkx2.1/TTF-1	Mouse	8G7G3/1	Invitrogen	18-0221	1:100
Nkx2.1/TTF-1	Rabbit	N/A	Seven Hills	WRAB-1231	1:1000
P63	Rabbit	H-129	Santa Cruz	sc-8344	1:100
Sox2	Rabbit	Polyclonal	Stemgent	09-0024	1:100
Sox2	Goat	Y-17	Santa Cruz	sc-17320	1:100
Pax 6	Rabbit	Polyclonal	Covance	PRB-278P	1:300
Pax8	Mouse	PAX8R1	Abcam	ab53490	1:100
EpCAM	Mouse, APC conjugated	EBA-1	BD Biosciences	BDB347200	1:100
Tuj1/Neuronal Class III β-Tubulin	Mouse	2G10	Sigma	T8578	1:4000
NGFR	Mouse	ME20.4	EMD Millipore	05-446	1:100
Mucin1	Armenian Hamster	MH1	NeoMarkers	HM-1630-P1ABX	1:200
Mucin5AC	mouse (Biotin)	45M1	Abcam	ab79082	1:100
Mucin5B	Rabbit	H-300	Santa Cruz	sc-20119	1:100
Mucin2	Rabbit	H-300	Santa Cruz	sc-15334	1:100
Foxj1	Mouse	2A5	e-bioscience	14-9965-82	1:100
CC-10	Goat	C-20	Santa Cruz	sc-9770	1:100
pro-SPC	Rabbit	Polyclonal	Seven Hills	WRAB-9337	1:2000
SPC	Rabbit	Polyclonal	Seven Hills	WRAB-76694	1:1000
mature SPB	Rabbit	Polyclonal	Seven Hills	WRAB-48604	1:1000
ABCA3	Rabbit	Polyclonal	Seven Hills	WRAB-70565	1:1000
HOPX	Rabbit	Polyclonal	Santa Cruz	sc-30216	1:250
Podoplanin	Rabbit	FL-162	Santa Cruz	sc-134482	1:100
AQP5	Goat	G-19	Santa Cruz	sc-9890	1:100

Supplementary table II: Quantitative Real-Time PCR primers

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
ACTB	TTTGAATGATGAGCCTTCGTGCCC	GGTCTCAAGTCAGTGTACAGGTAAGC
TBP	TGAGTTGCTCATACCGTGCTGCTA	CCCTCAAACCAACTTGTCAACAGC
Nkx2.1	CGGCATGAACATGAGCGGCAT	GCCGACAGGTACTTCTGTTGCTTG
Foxa2	TTCAACCACCCGTTCTCCATCAAC	TGTTCGTAGGCCTTGAGGTCCATT
Pax1	AAACCCTCCATGAACTGTCCTCTCC	CCCTGTGCTCCCTACTCCTACC
Pax8	ACTACAAACGCCAGAACCCTACCA	TGTCATTGTCACAGACGCCCTCA
Thyroglobulin	ACGGTTCCTCGCAGTTCAAT	GCAGCTTGGAACATAGGGGT
Foxp2	GCCTTGGCAGAGAGCAGTTTACCTTT	CCCGGACTACTGTTTCCATTGCTGT
Mash1	CGCGGCCAACAAGAAGATGAGTAAG	CATGGAGTTCAAGTCGTTGGAGTAGT
P63	CCTACAACACAGACCACGCACAGAA	CCTACAACACAGACCACGCACAGAA
Mucin5AC	GCACCAACGACAGGAAGGATGAG	CACGTTCCAGAGCCGGACAT
Foxj1	GGCATAAGCGCAAACAGCCG	TCGAAGATGGCCTCCCAGTCAAA
CC-10	TCATGGACACACCCTCCAGTTATGAG	TGAGCTTAATGATGCTTTCTCTGGGC
Podoplanin	AGGAGAGCAACAACTCAACGGGAA	TTCTGCCAGGACCCAGAGC
AQP5	GCCATCCTTTACTTCTACCTGCTC	GCTCATACGTGCCTTTGATGATGG
TSHR	TGCTGGACGTGTCTCAAACC	TAAGAAAGGTCAGCCCGTGT
SFTPA	GTGCGAAGTGAAGGACGTTTGTGT	TTTGAGACCATCTCTCCCGTCCC
SFTPB	TCTGAGTGCCACCTCTGCATGT	TGGAGCATTGCCTGTGGTATGG
SFTPC	CCTTCTTATCGTGGTGGTGGTGGT	TCTCCGTGTGTTTCTGGCTCATGT
SFTPD	TGACTGATTCCAAGACAGAGGGCA	TCCACAAGCCCTGTCATTCCACTT

Reference

1. **Gouon-Evans, V. et al. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nature biotechnology* 24, 1402-1411 (2006).**